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NuPsyllid: Rear and Release Psyllids as Biological Control Agents – An Economical and Feasible Mid-Term Solution for Huanglongbing (HLB) Disease of Citrus

Quarterly Report for the Period ending November 30, 2016

PROJECT DIRECTOR SUMMARY

Project Mission and Organization

The purpose of this NIFA-CAPS is to create attractive options for management of HLB by replacing the wild type insect vector (ACP) with a population that is unable to transmit the bacterial causative agent (CLas). Achieving this outcome will require progress in the following three areas of emphasis – An *Effector Mechanism*, A *Driver System*, and *Diffusion*. The current conditions threatening citrus production nationally require our key personnel to work concurrently on parallel technical plans and to accelerate the leading alternatives based on assessments by our team leaders, advisors and management. This research has established a broad foundational knowledge base of molecular interactions between host, pathogen and vector that is now contributing to additional NIFA-funded programs. Part of our outreach in the final phase of this program will be to integrate our progress with others focused on the HLB challenge and to extend the breadth of communications about new technologies for disease intervention.

To successfully use an Effector for insect replacement, we need to disrupt interactions required for the spread of HLB while adequately maintaining psyllid fitness. New actives discovered in this program that are specifically toxic to psyllids may be used for novel insect suppression technologies. While this is not the proposed population replacement, if genetic or other modes of conditional delivery can be developed then new forms of biological control will be feasible. For example, these assessments have suggested a near term application of this research for the protection of new solid block plantings from HLB. We continue to evaluate the “Psyllid Shield” control strategy. While it is not full insect replacement, it is based in part on research progress in the search for Effectors. CRDF has supplemented funding to model field results under various scenarios and has selected 5 RNAi sequences as field trial candidates based on the results of indoor experiments with caged insects. A key stakeholder partner identified by CRDF is investing in regulatory approvals necessary for field trials of this disease management concept.

Our team has updated project objectives and budgets for the remaining term of the funded work to synchronize our remaining cash flow with priorities set in the last Annual meeting.

TECHNICAL PROGRESS

Effector Mechanism

Initial assessments have not identified the required variation in CLas transmission to occur naturally in ACP populations. However the prospects for engineering a mechanism to achieve the desired phenotype are under active investigation. The effector is the content of the phenotypic change we aim to introduce. Candidate effectors are being identified through multiple parallel methods of investigation including bioinformatics, proteomics, yeast two-hybrid (Y2H), peptide-ligand and scFV-ligand libraries.

- There is a growing list of candidate effectors generated from bioinformatics (proteomic and transcriptomic), genetic (yeast two-hybrid) and physical methods (Far-Westerns--immunoprecepitations and mass spectrometry). This workflow of the Effector team has already generated more high quality targets than can be analyzed in bioassays. In many cases loss of gene expression through RNAi is highly toxic to psyllids. We have only conceived of two tools to use to disrupt the Effector Mechanism, RNAi and competitive protein ligand inhibitors (proteins, such as scFV antibodies or peptides). Secondary metabolites or RNA aptamers are potential additional options.
- Transcriptome expression profling: Extensive transcriptome data sets (the Transcriptome Computational Workbench; TCW) have been created from whole adults, adult salivary glands, adult guts, and nymphs infected or uninfected with CLas or CLso and are continually updated, with datasets associated with published manuscripts made available to the research community (www.sohomoptera.org/ACPPoP).
- Proteome expression profiling: During this report, TCW was updated to allow for protein sequence and corresponding spectral count data to be analyzed. In total (average of 3 technical replicates) 608 unique proteins were indentified by mass spectrometer in adult whole body, gut and salivary gland tissues. Results showed that 55% (304 proteins) had modified changes in abundance due to Liberbacter presence or absence in adult whole body, gut, and salivary gland tissues. The predicted functions of these proteins are were investigated using Gene Ontology and KEGG programs and results confirm that which we previously reported regarding our invasion model. To date 2 genes have been tested using RNAi, and data obtained within highlight an additional three genes as possible key players, which should experimentally investigated.
- Yeast two-hybrid: To date 47 ACP, 35 CLas and 10 phage genes have been selected for analysis. In total 25 ACP, 16 CLas, 6 phage, and 2 endosymbiont-associated genes were selected for further study. Those that showed interactions had variety of functions initially reported in virus-host pathogen systems, and more recently, for bacterial pathogen-interactions with their hosts.
- Co-immunoprecipitation: In total, roughly 25 gene candidates have been selected and/or attempted for CoIP analysis. To date 5 ACP, 2 CLas, and 2 phage genes have been tested (3 replicates) using crude protein samples extracted from both infected and uninfected adult whole body tissues. Results showed that a bacterial and phage protein interacting with psyllid genes involved in endocytic processes. Another phage gene was shown to interact with a psyllid transporter gene. Both psyllid genes were subjected to RNAi analysis and the bacterial and phage genes were subjected to

qPCR experiments. One manuscript using Y2H and CoIP findings is in preparation from this project, with an expected submission date of March 2017.

- TEM/SEM studies: Micrographs revealed putative endocytic/exocytic prosesses, biofilms that require complex cellular communication, and an intravesicular bacterial lifestyle evidenced for the first time allowing for additional effector candidates to be explored.
- Validation of effectors by RNAi testing has been conducted for 31 different psyllid genes using the single-gene and/or stacked (multiple-gene) RNAi approach and either CLso-infected (born and reared) or -uninfected (introduced to a CLso source plant) nymph and/or adult psyllids to date. Genes are selected as 'transmission interference candidates' by literature review of other pathosystems, from 3 different expression profiles e.g. proteome and transcriptome, and/or yeast-two hybrid analyses. Thus far, positive results (reduced transmission) have been obtained for 12 genes in functional transmission bioassays. In addition, 3 other genes have caused some psyllid mortality. Putative functions of the 12 genes strongly suggest an invasion model similar to other known bacterial pathogens. As a next step, additional genes are being selected for RNAi analysis based on these results.
- Quantitative PCR (qPCR) analyses have been conducted on 14 genes (previously reported 9) to date, including psyllid, phage and Liberibacter genes. Expressional profiles were obtained from early instars (1st -2nd), late instars (4th-5th), teneral, and non-teneral adult psyllids. One Liberibacter gene showed significantly higher relative expression (3-6x) in later instars to adult life stages. This gene is putatively involved in cell motility and also showed significant interaction in Y2H studies. Another Liberibacter gene, putatively involved in cell communication, showed a similar expression profile (high expression in late instars and adults). Interestingly a phage gene with a putative function implicated in virulence in other well-known pathosystems, was significantly higher expressed (2.5x) in the early instar. Low expression profiles for two phage genes with putative functions known to aid in virulence in other pathosystems, suggest that these are not key effectors in the CLas/ACP system. In addition, preliminary data for a unique psyllid gene predicted to be a key factor in invasion, shows differential expression between infected nymphs (1.3 fold increase) and adults (0.7- 0.03 fold decrease). Similar studies for 4 (previously reported 3) additional psyllid genes are in process both PoP and ACP to study relative gene expression in infected psyllids vs healthy psyllids. Work was competed to add 2 additional reference genes to add validity to the qPCR for the psyllid studies due the high level of variability in experimental conditions. An abstract "Psyllid vector-Liberibacter interactions at cellular and molecular interfaces" (Judith K. Brown, T.J. Rast, J.E. Cicero, and T.W. Fisher) was submitted to the "2017 International Research Conference on Huanglongbing".
- TCW databases, website, and bioinformatics interface: Database annotation updates are critical for completing the remaining transcriptome analyses needed to complete manuscripts anticipated during the last year of the project, and for annotating the comparative RNAseq data sets upon completion. Last quarter it was determined that quality of the data sets were not optimal because the computation of the read counts was not adequate. Therefore C. Soderlund and M. Willis worked together to determine appropriate parameters for the mapping. The TCW databases were rebuilt with the new data, given a December 2016 update archive. A study to determine candidate transcripts that influence psyllid speciation is ongoing. To date, orthologous pairs were computed using the TCW bi-directional best-hit (BBH) algorithm with e-value 1E-05

and the restriction that the alignment must overlap at least 50% of the shortest transcript, which resulted in 8892 pairs. A comparison of the 5'UTR, CDS, and 3'UTR showed percent similarity of 60, 73, 59 and percent GC-content of 24, 34.5, 23.5, respectively. The codon analysis shows an overall 24.4% synonymous codon and 33% non-synonymous pairs, where the synonymous codons were further broken down into 10.4% 2-fold and 13.4% 4-fold degenerate. The transition differences of the three codon positions are 9.3%, 6.7% and 18.6%; the transversion differences of the three positions are 9.8%, 8.7% and 16.0%. Using TCW, the alignments were written to file for analysis by the KaKs calculator program using the method=YN, and results loaded into TCW. There were 8850 with a p-value, where 7295 have KaKs<1 and 1553 have KaKs>1, with KaKs quartiles Q1 0.02873, Q2 0.11531, Q3 0.6708. The final objective is to map the Dc transcripts to the Dc genome sequence. Initial results showed multiple ambiguities when using blast results. A more strenuous approach was developed to use dynamic programming to get the best matches and data analysis is in progress.

- RNASeq time-course: 60 RNAseq ACP libraries were constructed from two treatments (ACP_CLas-infected vs uninfected), 5 stages (instar 1+2, instar 3, instar 4+5, teneral adult and adult) and 6 replicates, and these libraries were pooled with 12 libraries barcoded in a lane for 150 bp paired-end sequencing and 1,960 million reads totaling 296 G bp data were generated. The data assembly, mapping and annotation are in process.
- Endosymbiont genome sequencing: The psyllid ovary samples from 4 psyllid species; ACP, PoP-central, PoP-western and Carrot psyllid, were used for making 4 Illumina genomic libraries and pooled on a lane for 150 bp PE-sequencing. In total, 314 million reads were produced, and total bases are 47.4 G bp. The new longer read data will be integrated with the data we have (100 bp PE reads) to help improve the assembly qualities of the endosymbiont genomes. The assembly and annotation are in progress.
- Transcriptome and gene expression analysis of Asian citrus psyllid in response to *Ca.* Liberibacter asiaticus: We constructed RNA-seq libraries from *C*Las-infected and *C*Las-free ACP samples of three different developmental stages (nymphal instars 1-3, nymphal instars 4-5, teneral and post-teneral adults). With 150 bp paired-end sequencing on the Illumina Hiseq2500, we generated 152 Gb of sequence data from 56 million reads per library/replicate, which was assembled into 34,122 contigs with 18,827 (55.2%) being annotated, which were then further analyzed for potential functional classification and potential roles in infection. The results suggested that gene expression in different developmental stages did not respond in the same manner to *C*Las infection. With more contigs being up or down- regulated, nymphal instars 4-5 showed a more sensitive response to *C*Las infection than nymphal instars 1-3 and adults. A comprehensive analysis of the transcriptomes revealed vector life stage differences and differential gene expression in response to *C*Las infection, and identified specific genes with roles in nutrition, development, immune response and transmission pathways. There results were presented in March at the International Research Conference on Huanglongbing (IRCHLB) in Orlando, Florida, "Impact of *Candidatus* Liberibacter asiaticus infection on Asian citrus psyllid transcriptome" (Ruifeng HE, Mark WILLIS, Tonja W. FISHER, Carol A. SODERLUND, Kirsten PELZ-STELINSKI, Judith K. BROWN, David R. GANG).
- Two classes of peptides that may stop the Asian citrus psyllids ability to acquire/transmit (AcTrans) CLas have been identified in functional assays. One set of three hexameric peptides significantly reduced the psyllids subsequent ability to acquire/transmit (AcTrans blockers) the '*Candidatus*' Liberibacter asiaticus (CLas)

bacterium when fed to psyllid nymphs. We also identified two separate bactericidal peptides that kill CLas within infected leaf tissue when these peptides are taken up into the leaf vascular tissue (they are mobile in the leaf vascular tissue and reduce leaf bacterial titer by greater than 80% in 7 days). In the previous report, we presented preliminary results showing that by combining both AcTrans blockers and bactericidal peptides, we induced greater than 95% mortality in developing psyllid nymphs and none of the surviving nymphs have successfully acquired the CLas bacterium. Further replicated studies show similar results with high psyllid mortality and no complete acquisition (defined as movement into the salivary gland) of the bacterium by the surviving psyllids.

- We have initiated single AcTrans blocker peptide experiments in combination with the antimicrobial peptides to determine the minimal combination that has the desired effects on psyllid mortality and acquisition.
- We have developed a topical application strategy that could be deployable in the field and demonstrated its effectiveness in delivering organic antimicrobial molecules of MW of at least 600 MW and also effective in systemic delivery of dsRNA molecules of up to 300 bps (MW much larger than our peptides). We have initiated an order for large scale production of our active peptides (10g) which will be used in greenhouse scale proof-of-concept application experiments.
- We have developed a transgenic citrus strategy that will produce a single phloem-cleavable peptide that, when processed, produces the desired smaller and biologically active peptides (both AcTrans blockers and antimicrobial). This system utilizes an already identified peptide cleavage system within the phloem of citrus.
- Single chain antibodies targeting surface antigens on CLas have been created that interact with 12 different predicted surface epitopes. These antigens include the major outer membrane protein OmpA, two flagellar antigens, and the capsular polysaccharide synthase, and two pili components. Some of these have been expressed in transgenic citrus and others have been expressed and purified using a 6X histone tag strategy. These will be used for laboratory bioassays developed to study acquisition and CLas survival. Citrus rootstocks expressing two scFv have been made at Fort Pierce. A scFv selected to bind a surface exposed epitope of TolC = NodD (secretory pore) and a scFv selected against InvA, a protein produced by CLas believed to prevent apoptosis of infected cells. Multiple scFv selections have been introduced in citrus and multiple transformation events (~400 in all) are currently under evaluation for their effect on CLas survival in the plant, and acquisition/transmission by the psyllid.

Driver System

A new trait will not spread efficiently upon release within an existing population without a genetic bias of some kind. The driver is the medium of spread of the introduced phenotype--lack of CLas transmission. The drivers under investigation are viral, endosymbiont and chromosomal.

- From sequencing worldwide collections of *D. citri* and bioinformatics analysis, several potential candidate viruses have been discovered that might be useful for paratransgenesis delivery systems for inducing desirable traits in *D. citri*. Efforts continue to develop some of these for use as tools in this project.

- DcPLV was the first *D. citri* virus identified by us, in *D. citri* from Taiwan, China, and Brazil, but not yet from any U. S. collected *D. citri*. DcPLV is a novel insect virus with an unusual genome organization. DcPLV has a positive-sense ssRNA genome of 10,222 nucleotides and contains a single ORF coding sequence of 8,757 nucleotides. We have attempted to clone the entire genome obtained through the extension overlap PCR strategy as cDNA using different strains of *E. coli* cells including JM109, DHB10 and MDS but have not yet been successful. We hope to use cultured cells to recover infectious viruses. This bacterial free strategy was recently applied to create infectious forms of a virus in the genus *Flavivirus*.
- We have also expanded cell culture systems to aid in this work. Our hypothesis is that insect cells can offer a robust and efficient way to test reverse genetics forms of *D. citri* viruses. We are using *Drosophila melanogaster* S2 cells (Diptera), *Spodoptera frugiperda* sf9 cells (Lepidopteran), and BCIRL-AtE15-CLG, a hemipteran cell line from *Anasa tristis*. To optimize transfection systems, we used pMT-EGFP (homemade) and EGFP-mRNA (TriLink Bio Technologies) for DNA (plasmid) and RNA, respectively. As positive controls for transfection of our viruses, we are using two other insect viruses: a *Flock house virus* (FHV) infectious clone under control of the MT promoter (for plasmid inoculation), and a *Cricket paralysis virus* (CrPV) infectious clone under control of the T7 promoter for RNA (in vitro transcription) transfection. However, none of the cell lines mentioned above are known to be hosts for the *D. citri* viruses identified by us, thus negative results are hard to interpret. Therefore, we are attempting to develop psyllid cell lines for use with the *D. citri* viruses. Thus far, attempts to grow cells from embryos of both potato psyllids (*Bactericera cockerelli*) and Asian citrus psyllids (*Diaphorina citri*) have been made. Attempts to grow *B. cockerelli* cells in Hert-Hunter 70 (HH70) medium were conducted, with one replicate showing migration of cells from pulverized tissues, but subsequent cell growth and proliferation has yet to be observed. More recent attempts included attempting to grow *B. cockerelli* cells in LBM media, but no cell migration has so far been observed; regular observance of these cultures will continue. Similar efforts to culture *D. citri* cells in these same media have thus far yielded no positive results, but we will continue our efforts, increasing the number of starting eggs/embryos. One additional attempt to culture *D. citri* cells in Ate15A media, formulated for the culture of *Anasa tristis* cells, was made, but no cell migration has occurred. Potential reasons that the cells have not yet shown good ability to multiply could be that there are limited number of primary tissue culture explants left post treatment. One way to overcome this is to increase the number of embryonic fragments. Another possible method is to encourage attachment of more embryonic fragments to the bottom of the culture dish. We will attempt to screen more reagents/media to facilitate tissue sedimentation and adherence. Additionally, different media formulations and psyllid tissue-types will also be screened.
- One of the other viruses originally discovered by us early in our nuPsyllid work was *Diaphorina citri* Reo-like virus (DcRV). We have colonies of *D. citri* (Hawaiian collection) infected with DcRV in the UC Davis CRF. DcRV does not appear to induce obvious phenotypic effects in *D. citri* – HI, and our *D. citri* – CA lacks DcRV. We originally felt that DcRV would be too difficult to use for reverse genetics approaches and did not pursue using it. However, we have begun new efforts with DcRV. We previously failed to show DcRV transmission to naïve California *D. citri* by using on plant assays, and recent artificial diet assays also failed to show transmission to California *D. citri*. However, we recently transmitted DcRV from HI-*D. citri* (infected) to CA-*D. citri* (non-infected) psyllids by using an intra-hemocoel micro-injection approach. We also are

screening offspring to see if DcRV can be transmitted sexually between CA- and HI-*D. citri*. We are now evaluating DcRV potential for inducing negative phenotypes and affecting *D. citri*, but also to determine if it might interfere or have any effects on the ability of *D. citri* to transmit *C*. Liberibacter asiaticus to plants.

- With respect to the bacterial driver system a goal for the project going forward is to procure ST-173 *Wolbachia* for evaluation in CLas transmission experiments. Because this entails artificially infecting *Wolbachia*-free *D. citri* for comparison against ST-FL *D. citri* (wDi), we have pursued developing a robust *Wolbachia*-free colony. Previous efforts to eliminate wDi with antimicrobials used in aphid model systems were insufficient to reduce *Wolbachia* cultures. Antibiotic sensitivity testing of chloramphenicol and kanamycin with isolated wDi and wDi cell lines was conducted to determine the efficacy of these compounds against *Wolbachia* prior to introducing it into *D. citri* diet. Briefly, bacterial cells were isolated from the cell line (isolated *wDi* from S2 (180mL; $6.6x10^6$ cells/mL). At Day 0, wells of a 96 well plate were inoculated with 0.5 mL of isolated *wDi* plus 1.5mL of S2 cell culture medium, and treated with antibiotics. On days 1, 2 and 3, each sample was assayed for cell mortality using BacLight live-dead staining. Absolute quantification PCR (standard curve method) was used to determine *ftsZ* copy number (ftsZ is an essential cell division protein in *wDi*). The concentration of each compound yielding 100% reduction in wDi after 3d is currently being valuated in artificial feeding bioassays with *D. citri.* Transinfections with non-native hemipteran *Wolbachia* will begin next month. Transmission capacity will subsequently be evaluated between D. citri isolines that are 1) infected with native wDI, 2) co-infected with wDi and non-native *Wolbachia* (established previously in this project), and 3) infected with non-*Wolbachia*.
- During the past quarter, we have continued efforts to develop protocols for successful microinjection of ACP embryos in conjunction with Dr. Al Handler, though survival and fertility of hatched nymphs remains very low. Survival of *D. citri* following egg collections have been improved by manipulating eggs while still on intact flush. For this method, citrus shoots with flush containing eggs are excised from the plant prior to injection. Eggs are manipulated under a stereomicroscope, and then the seedlings are placed in an eppendorf tube sealed with parafilm until nymphs emerge. Flush is stored in an incubator at 25C, 80% RH, 16:8 L:D photoperiod. Following hatch, the nymphs transferred to fresh flush.
- Efforts continue to streamline the process for generating insects that carry reciprocal translocations to facilitate a chromosomal gene drive system for population replacement in the psyllid. Several chromosome translocation-based drive elements have been generated in Drosophila. A manuscript describing how to generate chromosome translocations, their predicted drive properties, and their actual properties been posted online at bioRxiv.org, http://biorxiv.org/content/early/2016/11/17/088393. Our original work used a three transgene approach that required a number of generations and crosses to generate translocations. We have now generated a number of translocations in Drosophila using a two transgene approach that involves two strains, one expressing Cas9 and a second line expressing a guide RNA that cleaves both transgene-bearing chromosomes. At least one of these seems to be driving.
- Caution must be used in making the determination that any one of the lines scored as positive for being a translocation based on marker rescue with the Cas9 system. This is because, for still unclear reasons (being explored using PCR and sequencing), the act of cleavage sometimes results in marker activation that does not correspond to generation of an actual translocation. We discovered this in several drive experiments.

Ectopic marker activation in the absence of translocation seems to be the cause. We have found that the only way to ensure that a putative translocation is really a translocation is to outcross heterozygotes and look for the 50% embryo lethality that must occur if the translocation is present. This is then followed by PCR to confirm.

- We are also still pursuing a third approach, in which we try to generate a translocation directly in the germline of an injected embryo, as this would simplify the process even further. We have had success in bringing about homologous integration of one of the two constructs in an injected embryo, and are working to identify conditions that allow a second construct to integrate at the same time, in the proper orientation. We know that both fragments are integrating, but one of them seems to be integrating in the wrong orientation. In addition, as noted above, a number of false positive have been identified. We are trying to understand the basis for these since eliminating them will make identification of true translocations much easier.
- We have also begun the process of working to generate psyllid cell lines. This approach involves trying to simplify the process of immortalization by taking cells from embryos and introducing oncogenes and cell death inhibitors, and/or inactivating tumor suppressor genes. These approaches can allow cells to survive and proliferate even in the absence of some growth regulators that would normally need to be present, but that are unknown.
- We have spent the last quarter generating a number of constructs designed to either overexpress or knockdown or knockout the expression of specific genes involved in cell death and cell proliferation using psyllid-derived reagents. For overexpression we are using sequences that should encode the psyllid ubiquitin promoter or the baculovirus IE3 promoter. For the Crispr/Cas9 targeting of specific genes we are using fluorescent reporters under the control of a ubiquitin promoter, linked to homology arms for the genes being targeted, components of the hippo/warts pathway.
- We are also continuing our efforts to generate transgenics using adult injection, thus far without success. Early efforts focused primarily on females. In the last cycle we initiated work on males, which is continuing.

Diffusion

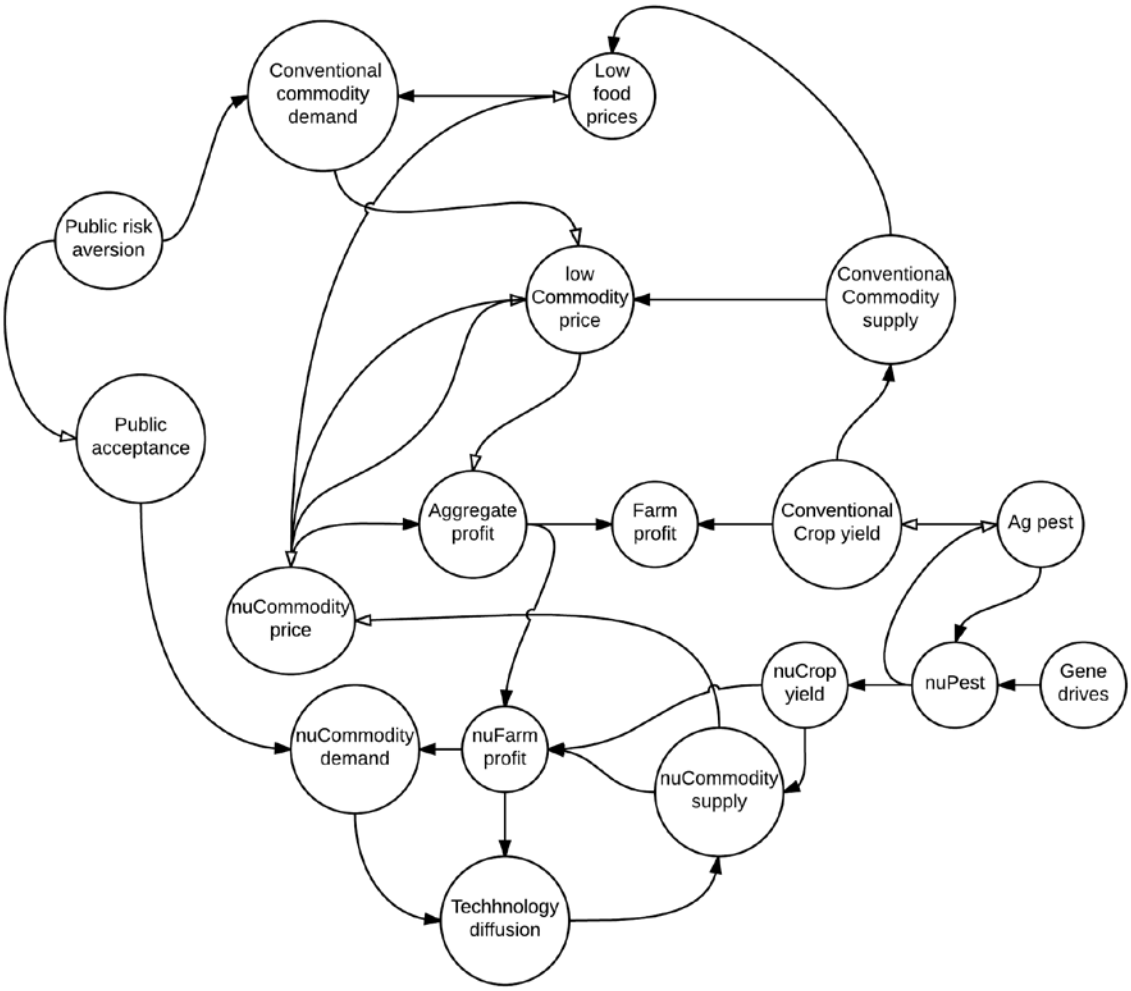
Once a nuPsyllid population is developed, its successful use will depend on series of factors based on the overall phenotype and fitness of the population in the environment and most importantly, will depend on human adoption, including the behavior of regulatory agencies, growers and consumers. All of these attributes must be modeled accurately for a nuPsyllid release to be used effectively. As for any other innovation, diffusion is the rate of change. Several aspects of the technical and communication plan can be addressed most effectively only when an actual candidate nuPsyllid is available for release. The ability to rear, release and monitor psyllids has been initiated and is of immediate use in HLB disease management applications outside of this proposal.

- There is a substantial effort to rear and release any type of nuPsyllid under development:
    - Florida, Texas, and California will each develop and maintain its own colony to provide nuPsyllids for initial greenhouse studies and pilot field releases within its borders. The decision as to where to house nuPsyllid colonies within each state will be likely have to be made at several administrative levels.

    - Regulatory agencies will likely require that nuPsyllid colonies be housed in a controlled/quarantine facility. Potential sites in each state were identified.
    - An estimated population size for a nuPsyllid required for testing cannot be provided until the driver mechanism is selected. The effector mechanism may have associated fitness costs, as well, and these will have to be figured into rearing effort estimates.
    - The initial plan is to piggyback nuPsyllid rearing efforts onto that of the existing parasitic wasp programs (*Tamarixia*) for initial testing with care to control for *Tamarixia* contamination.
- Induction of foliar volatiles: The development of 'super-stimuli' which are strong behavioral elicitors, may provide a means of boosting the efficacy of synthetic attractants by enabling them to outcompete background stimuli. Plant pathogens elicit the production of super stimuli in their host plants to make infected plants more attractive to insect vectors; examination of pathosystems may reveal the identity of potentially useful super-stimuli. Of significance to the Las-ACP-citrus pathosystem, Dr. Lukasz Stelinski (UF) and his associates have shown that Las-infected foliage emits the volatile signaling compound methyl salicylate, and that it acts as a super-stimulus in attracting uninfected ACP to Las-infected trees. The emission of methyl salicylate (MeSA) is governed by the production of salicylic acid (SA), an internal signaler that is induced by pathogen infection. The Stelinski and Sétamou (TAMU) labs are developing scent attractants containing methyl salicylate.
- Another important attack/stressor signaler system in plants is the jasmonic acid/methyl jasmonate (MeJA) system. We showed that exogenous application of MeJA to potted Las- and Las+ Valencia orange trees significantly altered volatile emission both quantitatively and qualitatively. In behavioral assays, ACP significantly aggregated at higher levels of MeJA-treated foliage.
- We also showed that exogenous applications of salicylic acid to Las- and Las+ Valencia trees resulted in: 1) Emission of a quantitatively greater amount of volatiles; 2) Production of high levels of MeSA, with this compound comprising 50% of the total amount of volatiles emitted; and 3) Absence of indole, E-jasmone and other compounds in the foliar odor induced by the application of MeJA.
- A study is underway to determine whether an emulsified wax carrier (SPLAT, ISCATech, Inc.) can be used to convey MeJA to citrus foliage. One of the primary aims of this study is to determine the duration of the expression of foliar volatiles induced by exposure to MeJA. We will also devise optimal loading levels for achieving a maximal response from the foliage.
- Our initial results indicate that applying individual droplets of SPLAT containing MeJA to foliage does not induce defensive volatiles unless the MeJA concentration is relatively high; i.e., 90 mM MeJA/SPLAT droplet. Likewise, introducing air that had passed over SPLAT with 90 mM MeJA into a volatile sampling vessel did not induce a volatile response in test trees. In both tests, volatile MeJA was recovered from the collection vessels. Interestingly, a high level of response was achieved using a low viscosity SPLAT formulation containing only 10 mM MeJA that was sprayed on the foliage. We are not sure why coating the foliage with MeJA is necessary to induce the production of defense volatiles while the mere presence of MeJA in the collection vessel does not induce their production. We will continue to investigate this phenomenon.
- Studies will soon be underway to: 1) Determine the effect of SA- and MeJA-treatment on ACP colonization; 2) Identify the volatiles emitted by SA- and MeJA-treated foliage

that influence ACP behavior; 3) Develop ACP scent attractants based on SA- and MeJA-treated foliage.

- Field trials of ACP scent attractants using DPI 3D psyllid traps: Field trials are being conducted in southern California by UC Riverside and USDA-ARS with the 3D traps made by the Florida Department of Plant Industry. Results show that two scent bait mixtures supplied by us, one that mimics lemon and the other orange jasmine, increase trap captures by 20 to 30%. Since these traps are designed to preserve ACP for genetic testing, this result is significant because it shows the potential of improving scent baits for monitoring nuPsyllid.
- Statistical analysis of volatile emission of Las- and Las+ trees treated with MeJA versus unsprayed control trees indicated that only a few volatiles are involved in driving most of the differences observed between treatments. Formulations containing these driver volatiles will be field tested to determine if they can function as effective scent lures for ACP.
- The sustainability/economics/modeling team continued to develop analytical infrastructure based on the generic needs to understand system dynamics and potential impacts of technology adoption.
- The economics team (UoW) completed an analysis of economic surplus achievable from eradication of HLB in the Florida industry. The analysis, which is based around supply shifts and price changes since the arrival of HLB, indicates that the total saving in consumer/producer surplus that would be obtained from removal of HLB is in the order of $500M per annum.
- Two members of the team (McRoberts and Mitchell) presented keynote papers at the IRCHLB.
- The analysis of technology sustainability was focused on completing a draft of a cognitive map (causal diagram) associated with the introduction of gene-drive technology for control of agricultural pests. Dynamic analysis of the draft cognitive map shown below is in progress:



- The outreach team conducts monthly teleconference calls to select and discuss projects that showcase research programs addressing the HLB problem. With funding from other agencies the subject matter has been expanded beyond the nuPsyllid program, with the goal of educating growers about new technologies (both genetic engineering and non-GE) for managing HLB. The Science for Citrus Health web site http://ucanr.edu/sites/scienceforcitrushealth/ was made public this quarter. It is divided into four sections: 1) Early Detection Techniques, 2) Strategies for Established Orchards, 3) Strategies that Require Replants, and 4) nuPsyllid. The team has posted the following research updates and additional research updates are planned for the next quarter.
- Early Detection: Cristina Davis; Using volatile changes in citrus and Ali Pourezza; Starch accumulation sensor
- Established Orchards: Michelle Cilia; Managing psyllid gut cells to block transmission of CLas and Lukasz Stelinski, Nabal Killiny; Using interference RNA to manage ACP
- Replants: Jim Thomson; Founder lines used to improve HLB tolerance
- nuPsyllid: Kirsten Pelz-Stelinski; Altering the Asian citrus psyllid's beneficial bacteria to stop HLB spread
- A poster entitled "Resources for reaching out to growers and end-users on approaches to combating citrus greening disease" highlighting the web site was presented by

Peggy Lemaux at the IRCHLB. Postcards announcing the web site were provided to poster visitors at the meeting.

SUMMARY

There are a number of excellent candidate effector targets including several identified in a functional screen. It would be ideal to test these candidates in a psyllid viral vector.

The combined data strongly suggest an “invasion model” in which CLas/CLso transforms the endocytic/exocytic host pathways to facilitate internalization, infection, and circulation in the psyllid host and vector. Briefly, a model involving a putative phage gene that acts as an effector, which may operate in conjunction with a unique ACP gene to alter the function of genes associated with clathrin-mediated endocytosis, actin cytoskeletal rearrangements, and vacuolar formation, and exocytosis.

The translocation driver system is ready if the transformation bottleneck can be overcome. Because of the progress with the effector characterization and driver options, it is an important time for the team to continue to:

- select and prioritize effectors;
- obtain antibody reagents for top effector candidates;
- use the bioassay platform for comparative testing of the phenotypes in ACP, maximizing transmission blockage and minimizing fitness loss;
- accelerate development of the DCPLV vector and be prepared to use others that might be immediately useful for effector prioritization;
- analyze the phenotypes of both native and non-native *Wolbachia* introduced into ACP;
- determine if *Wolbachia* transformation is a feasible goal;
- develop ACP transformation capacity at any level of efficiency;
- continue to ready the engineered translocation constructs;
- begin to model the logistics of rearing and releasing nuPsyllid around hypothetical specifications and explicit assumptions;
- optimize trap design for monitoring and other potential control applications;
- engage the grower community in a broad educational outreach to raise awareness of the alternatives for genetic technologies in the management of HLB
- provide support and continuity as additional teams are funded that can build these results into existing and pending research programs seeking HLB solutions.